Cleavage targets and the d-arginine-based inhibitors of the West Nile virus NS3 processing proteinase

Sergey A. SHIRYAEV*,1 Boris I. RATNIKOV*†, Alexei V. CHEKANOV*‡, Sergey SIKORA*, Dmitri V. ROZANOV*, Adam GODZIK*, Jun WANG*, Jeffrey W. SMITH*, Zhiwei HUANG*, Iris LINDBERG†, Melanie A. SAMUEL‡, Michael S. DIAMOND‡ and Alex Y. STRONGIN*‡

*The Burnham Institute, La Jolla, CA 92037, U.S.A., †Louisiana State University Health Sciences Center, New Orleans, LA 70112, U.S.A., and ‡Departments of Medicine and Molecular Microbiology, Washington University Medical School, St. Louis, MO 63110, U.S.A.

Mosquito-borne WNV (West Nile virus) is an emerging global threat. The NS3 proteinase, which is essential for the proteolytic processing of the viral polyprotein precursor, is a promising drug target. We have isolated and biochemically characterized the recombinant, highly active NS3 proteinase. We have determined that the NS3 proteinase functions in a manner that is distantly similar to furin in cleaving the peptide and protein substrates. We determined that aprotinin and D-arginine-based 9–12-mer peptides are potent inhibitors of WNV NS3 with IC₅₀ values of 26 nM and 1 nM respectively. Consistent with the essential role of NS3 activity in the life cycle of WNV and with the sensitivity of NS3 activity to the D-arginine-based peptides, we showed that nona-D-Arg-NH₂ reduced WNV infection in primary neurons.

We have also shown that myelin basic protein, a deficiency of which is linked to neurological abnormalities of the brain, is sensitive to NS3 proteolysis in vitro and therefore this protein represents a convenient test substrate for the studies of NS3. A three-dimensional model of WNV NS3 that we created may provide a structural guidance and a rationale for the subsequent design of fine-tuned inhibitors. Overall, our findings represent a foundation for in-depth mechanistic and structural studies as well as for the design of novel and efficient inhibitors of WNV NS3.

Key words: Dengue virus, flavivirus, furin, peptide inhibitor, proteinase, West Nile virus.

INTRODUCTION

WNV (West Nile virus) is a member of the Flaviviridae family and it was first isolated in 1937 in the West Nile district of Uganda [1]. WNV is transmitted to animals including humans, through mosquito bites. Since 1999, when the virus was identified in the U.S.A., the virus has spread rapidly and has infected the order of 15,000 people and caused more than 600 deaths. There is currently no effective vaccine or antiviral drug to protect against WNV infection [2].

WNV has a single strand, positive polarity, RNA genome. The 11 kb RNA genome codes for a single polyprotein precursor. The precursor comprises three structural proteins [C (capsid), M (membrane) and E (envelope)] and seven NS (non-structural) proteins arranged in the order C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5. Post-translational proteolytic processing of the WNV polyprotein precursor is required to produce the functional viral proteins which assemble a new viral progeny [3]. This processing is performed by host furin-like proprotein convertases and viral serine protease encoded by the N-terminal 184-residue portion of NS3 [4,5]. Host proteinases are responsible for cleavage at the C/prM, prM/E, E/NS1, NS1/NS2A and NS4A/NS4B junctions as well as for cleavage within prM at a late step during virion morphogenesis. Work by several groups suggests that the flaviviral NS2B–NS3 proteinase participates in cleavage at the NS2A/NS2B, NS2B/NS3, NS3/NS4A and NS4B/NS5 junctions, as well as at the internal sites within C, NS3 and NS4A [6–13]. The NS3-sensitive cleavage sites in the polyprotein precursor predominantly exhibit arginine at the P1 position, arginine and lysine at the P2 position, and glycine and serine at the P1′ position. These features suggest that NS3 is similar, although less restricted, in its cleavage preference when compared with furin, which cleaves predominantly after an RXR/KR↓ motif. Because mutation of the NS3 cleavage sites in the flaviviral polyprotein precursor abolishes virus infectivity [6,9,13–17], the catalytically active NS3 is essential for the life cycle of WNV and similar flaviviruses. The functional significance of the NS3-mediated processing in the life cycle of WNV makes this proteinase an attractive drug target for antiviral therapies [18].

The sequence of the WNV NS3 protease domain with a functional catalytic triad (His₃⁰¹, Asp₇₅⁰, Ser₁₃₅) is approx. 50% identical with the NS3 of Dengue virus. The spatial structure of the Dengu and hepatitis C NS3 is known, which provides an opportunity for in silico modelling of the WNV protease structure [19]. By analogy with the Dengu NS2B–NS3 tandem, the viral protein NS2B is believed to be an activator of the NS3 WNV enzyme. The 40-residue hydrophilic central domain of NS2B is sufficient to activate the catalytic activity of the NS3 protease [8,12,20,21]. Despite the fact that NS3 autocatalytically cleaves at the NS2B/NS3 junction, it remains controversial as to whether NS2B is absolutely required for NS3 activity [18,22]. Although the viral targets of NS3 protease have been studied in substantial detail, it remains unknown whether NS3 also cleaves host proteins, and whether these cleavages contribute independently to cellular injury and pathogenesis.

In the present paper, we describe the enzymatic characteristics of a catalytically potent WNV NS3 proteinase construct and the potential function of the NS2B sequence. Using this information,
we identified low-nanomolar range inhibitors of the WNV NS3 cleavage activity. Collectively, our experiments provided a foundation for the in-depth studies of WNV NS3 and, potentially, for the development of a broad-range inhibitor. We believe that, following a structural optimization, these inhibitors will also be effective against several other clinically relevant members of the flavivirus family.

### MATERIALS AND METHODS

#### Reagents

Aprotinin, N-p-tosyl-L-Lys-CHO, the inhibitors of cathepsin B [L-3-trans-(propylcarbamoyloxirane-2-carbonyl]-L-Ile-L-Pro and [L-3-trans-(propylcarbamoyloxirane-2-carbonyl]-L-Ile-L-Pro methyl ester, and the Boc-QAR-AMC (Boc is t-butoxycarbonyl and AMC is 7-amino-4-methylcoumarin) and Boc-O-benzyl-EGP-pNA (pNA is p-nitroanilide) peptide substrates were purchased from Sigma. Serpins (α1-antitrypsin and α1-antitrypsin variant Portland), the Z-RR-AMC (Z is benzylalcohol) peptide substrate, and the Boc-d-R-tosyl-OL and dec-RVKR-cmk (dec is decanoyl and cmk is chloromethylketone) inhibitors were obtained from Calbiochem. The Boc-RRV-R-AMC, Pys-RytkR-AMC (Pys is pyroglutamic acid) and Boc-APG-R-AMC peptide substrates were acquired from American Peptide. MBP (myelin basic protein) was obtained from Bioventures. Plasmid construction and cloning

The cDNA sequence of the pathogenic New York strain of WNV was used as a template to generate the NS2B–NS3 construct. WNV cDNA encoding NS2B and NS3 was provided by Dr Richard Kinney (Centers for Disease Control and Prevention, Fort Collins, CO, U.S.A.). NS3 was generated by PCR using 5′-GGCCGGGAGGATGTTGGATGAGCGGGGGGACACTCCC-3′ as direct and reverse primers respectively. The resulting PCR products were amplified to obtain the nona-peptide linker GGGGSGGGG (underlined in the primer sequence). The PCR products were purified and cloned into the pET101 Topo cloning vector, with the Boc-Arg-AMC substrate, the rate of substrate hydrolysis increasing concentrations of the inhibitors. Following addition of the Boc-Arg-AMC substrate, the rate of substrate hydrolysis was monitored continuously at λ_{ex} (excitation wavelength) of 360 nm and λ_{em} (emission wavelength) of 465 nm on a Spectramax Gemini EM fluorescence spectrophotometer (Molecular Devices). All assays were performed in triplicate in wells of a 96-well plate. The D-arginine peptide inhibitors (hexa-, hepta-, octa-, nona-, deca-, undeca- and dodeca-D-Arg-NH2) were synthesized by solid-phase peptide synthesis. All peptides were C-terminally amidated, with free N-termini. The actual molar concentration of the peptides was lower because of the presence of water and trifluoroacetate salt in the samples. Based on kinetics and active site titration studies, we estimate that the samples contained approx. 25–35% of actual peptide. The concentrations reported in the Results section were calculated using these corrections.

#### Enzyme expression and purification

For protein purification, cells were resuspended in 20 ml of lysis buffer (PBS containing 1 M NaCl, 10 mg/ml lysozyme and protease inhibitor cocktail). The cells were disrupted by sonication on an ultrasound disintegrator. The pellet was removed by centrifugation at 20000 g for 30 min. The recombinant NS2B–NS3 construct, C-terminally tagged with a hexahistidine tag, was purified from the soluble fraction by affinity chromatography on a Co2+-chelating Sepharose Fast Flow 15 cm column (Amersham Biosciences) equilibrated with PBS, 1 M NaCl and 1 mM PMSF. After washing the column extensively with PBS, 1 M NaCl and 1 mM PMSF, the bound proteins were eluted with 60 ml of a 10–500 mM imidazole gradient. Fractions (1.5 ml) were collected and analysed by SDS/15%-PAGE. The NS2B–NS3-containing fractions were pooled and dialysed against 10 mM Tris/HCl buffer, pH 8.0, containing 0.005% Brij 35. The dialysed material was then incubated for 16 h at 24°C to allow the autolytic conversion of the NS2B–NS3 construct into the individual NS3 enzyme. The resulting NS3 samples were re-checked by SDS/PAGE to confirm that autolytic conversion had been completed.

#### Synthesis of peptide inhibitors

The D-arginine peptide inhibitors (hexa-, hepta-, octa-, nona-, deca-, undeca- and dodeca-D-Arg-NH2) were synthesized by solid-phase peptide synthesis. All peptides were C-terminally amidated, with free N-termini. The actual molar concentration of the peptides was lower because of the presence of water and trifluoroacetate salt in the samples. Based on kinetics and active site titration studies, we estimate that the samples contained approx. 25–35% of actual peptide. The concentrations reported in the Results section were calculated using these corrections.

#### Protease assays with fluorogenic peptides

The assay for NS3 protease activity was performed in 10 mM Tris/HCl buffer, pH 8.0, containing 20% (v/v) glycerol and 0.005% Brij 35. The substrates and enzyme concentrations, unless indicated otherwise, were 24 μM and 10 nM respectively. The total assay volume was 0.1 ml. Initial reaction velocities were monitored continuously at λ_{ex} (excitation wavelength) of 360 nm and λ_{em} (emission wavelength) of 465 nm on a Spectramax Gemini EM fluorescence spectrophotometer (Molecular Devices). All assays were performed in triplicate in wells of a 96-well plate. The K_{m} and k_{cat} values were derived from a double-reciprocal plot of 1/V_{0} against 1/[S], using the Lineweaver–Burk transformation: 

\[ \frac{1}{V_0} = \frac{K_m}{V_{max}} \times \frac{1}{[S]} + \frac{1}{V_{max}} \]

where V_{0} is the initial velocity of substrate hydrolysis, [S] is the substrate concentration, V_{max} is the maximum rate of hydrolysis, and K_{m} is the Michaelis–Menten constant. The concentration of the catalytically active proteinase was measured using the fluorescent assay by titration against a standard aprotinin solution of known concentration. The concentration of active NS3 was close to 100% when compared with the protein concentration.

For the determination of the K_{i} value of the inhibitors, NS3 was pre-incubated for 60 min at ambient temperature (18°C) with increasing concentrations of the inhibitors. Following addition of the Boc-RRV-R-AMC substrate, the rate of substrate hydrolysis was monitored as described in the preceding paragraph. For the inhibitors with the apparent inhibition constants K_{i} (app) value below the enzyme concentrations, the K_{i} (app) was derived from non-linear regression fits of a against [I]_{0}, using the following equation:

\[
a = 1 - \left( \frac{([E]_0 + [I]_0 + K_i) - [([E]_0 + [I]_0 + K_i)^2 - 4[E]_0[I]_0]}{2[E]_0},
\]

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where $a = V_i / V_{o}$, $V_i$ is the initial velocity at the inhibitor concentration, $V_{o}$ is the initial velocity in the absence of the inhibitor, $[E]_0$ is the total enzyme concentration, and $[I]_0$ is the total inhibitor concentration. For the inhibitors with the $K_i$ (app) value significantly above the $[E]_0$, the $IC_{50}$ was derived by fitting the $V_{o}$ against log $[I]_0$ plots with sigmoidal dose–response curves. For the competitive inhibitors, the inhibition constant ($K_i$) was derived using the Cheng–Prusoff equation:

$$K_i = IC_{50}/(1 + [S]_0/K_m).$$

Cleavage of protein targets

PA83 (2 µg, 1.2 µM) was co-incubated with NS3 (1.7 µg; 2.5 µM) for 4 h at 37 °C and then analysed by SDS/15 % PAGE. MBP (11 µM) was co-incubated with NS3 (44–440 nM; an enzyme/substrate ratio of 1:25–250) for 60 min at 37 °C. Where indicated, dodeca-D-Arg-NH$_2$ (2–200 µM) and aprotinin (20–120 µM) were each added to the reactions. The digested samples were analysed by SDS/PAGE using 4–20 % polyacrylamide gels.

Cleavage of peptides and MS analyses

The peptides derived from the sequence of WNV prM (K$^{TRHSRRSSRSL^{38}}$) which span the furin cleavage site (underlined) and from the anchored capsid C protein sequence (A$^{INRD^{20}}$STKQKRGG$^{33}$) that includes the known NS3 cleavage site (underlined), were obtained from GenScript. In the C protein peptide, Asp$^{98}$ (italicized) was substituted for Arg$^{98}$ of the original sequence. Unless the R98D mutation is made, the cleavage of the peptide will occur at both the RR$^{59}$ and the KR$^{49}$ sites of the original sequence. The peptides (1 µg; approx. 30 µM) were incubated with NS3 (0.7 µg, 1.25 µM) for 2 h at 37 °C in 20 µl of 10 mM Tris/HCl buffer, pH 8.0, containing 20 % (v/v) glycerol. The molecular mass of the intact peptides and the digest products was determined by MALDI-TOF (matrix-assisted laser-desorption ionization–time-of-flight)–MS analysis.

Cell-based inhibitor assays and neuronal cultures

Primary sympathetic neuronal cultures were generated from SCG (superior cervical ganglia) of post-natal day 1 C57BL/6f mice [24]. Dissected SCG were treated sequentially with 1 mg/ml collagenase (Worthington) for 30 min at 37 °C, and then with trypsin (Sigma) for 30 min at 37 °C. Neurons were dissociated by gentle pipetting and were seeded in wells of an eight-well chamber (Nunc) coated with rat tail collagen (6000 cells/well). SCG neurons were cultured for 3 days in AM50 medium, which contained minimal essential medium supplemented with 10 % heat-inactivated FBS (foetal bovine serum), 2 mM L-glutamine, 30 µM fluorodeoxyuridine, 30 µM uridine, 50 ng/ml NGF (nerve growth factor) (Harlan Bioproducts) and 3.3 ng/ml aphidicolin (Fisher Scientific). Neurons were subsequently maintained in aphidicolin-free medium. Experiments were performed using neurons cultured for 7 days before infection.

Primary SCG neurons were treated with increasing concentrations of nona-D-Arg-NH$_2$ in serum-free medium for 2 h before infection with the pathogenic New York strain of WNV at a multiplicity of infection of 1 for 1 h at 37 °C. Cells were infected in the presence of nona-D-Arg-NH$_2$. Free virus was then removed by serial washing with PBS followed by a 1 h wash with serum-containing medium. After an additional 2 h of treatment with nona-D-Arg-NH$_2$ in serum-free medium, serum was added to the cells. Medium samples were taken 24 h after infection. The virus titre in the samples was determined by plaque assays on BHK21 cells [25].

In silico modelling

A homology search for the WNV NS3 sequence from the MEROPS Peptidase Database at http://merops.sanger.ac.uk (accession number MER00288) was performed using PSI-BLAST on the NCBI BLAST server (http://www.ncbi.nlm.nih.gov/BLAST/). A multiple sequence alignment was performed using the NS3 sequence of Kunjin virus (accession number MER00287), Murray Valley encephalitis virus (accession number MER00291), Japanese encephalitis virus (accession number MER00293), Dengue type 1 virus (accession number MER-04078), Dengue type 2 virus (accession number MER00282), Omak haemorrhagic fever virus (accession number MER35167), tick-borne encephalitis virus (accession number MER02975), Louping ill virus (accession number MER02434), Alkhurma virus (accession number MER16976), Langat virus (accession number MER04116), Powassan virus (accession number MER04117), Deer tick virus (accession number MER25135), yellow fever virus (accession number MER00290) and Usutu virus (GenBank® identifier gi|45378910). The alignment was performed using the T-COFFEE software (T-COFFEE, version 1.37; Swiss Institute of Bioinformatics; http://www.ch.embnet.org/software/TCoffee.html). The resulting alignment was plotted using the GeneDoc software (GeneDoc, version 1.0, Free Software Foundation; http://www.psc.edu/biomed/genedoc/). The results of the multiple sequence alignment were also used to generate a phylogenetic tree. The tree was created using Mega-2 software (Molecular Evolution Genetics Analysis, Version 2.1, Penn State University; http://www.megasoftware.net/). An initial distance estimation was performed using the Kimura correction. Tree topology was derived using the neighbour-joining method. The values obtained from each bootstrap analysis are shown for the branches.

Modelling of WNV NS3 was performed using the Modeller and Swiss PDB Viewer software, and the Dengue NS3 protease (PDB code 1DF9) as a template. The model was validated using PSQS (Protein Structure Quality Score; http://www1.jcgs.org/qsqs) and ProCheck (version 3.5.4, EBI, Cambridge; http://bioclic.uki.ac.uk:8400/). The model total energy score is $-0.3739$ (local $-0.0524$, burial $-0.0013$, contact $-0.0158$), which is consistent with the feasible molecular models. The R.M.S.D. (root mean square deviation) value of the model is 0.21 Å (1 Å = 0.1 nm). This value is an indication of a highly reliable model. The structural images of WNV NS3 and furin (PDB code 1P8J) were prepared by using PyMOL (version 0.95, Delano Scientific; http://pymol.sourceforge.net/). Surface residues of WNV NS3 were determined using a surface-mapping analysis [26].

RESULTS

Purification and autolytic conversion of NS2B–NS3 into NS3

Previous studies showed that the presence of either the full-length NS2B sequence or the 40-residue central NS2B domain linked to the N-terminus of NS3 significantly enhanced the accumulation of the soluble recombinant NS3 in E. coli [18,22]. We used a similar approach to express the WNV NS3. The 40-residue central portion of NS2B (short NS2B) was linked with NS3 via a GGGGSGGGG linker. To facilitate its isolation, the NS2B–NS3 construct was C-terminally tagged with a His$_6$-tag. To characterize the catalytic activity of NS3, we expressed the short NS2B–NS3 construct in E. coli. After induction with IPTG, large amounts
Figure 1  Constructs and purification of the NS3 proteinase of WNV

(A) The 40-amino-acid-long central portion of NS2B (short NS2B) was linked with NS3 via a GGGGSGGGG linker. A construct was C-terminally tagged with a His6-tag. After its isolation, the short NS2B–NS3 construct autolytically cleaved the K15↓G16 scissile bond (numbering starts from the N-terminus of NS3) and generated the individual NS3 proteinase. The constructs had a C-terminally tagged His6-tag. The presence of the His6-tag facilitated the purification of the constructs by metal-chelating chromatography. (B) The His6-tagged NS2B–NS3 construct was cloned in the pET101 plasmid and expressed in E. coli. The soluble construct was purified from E. coli by Co2+ metal-chelating chromatography. The construct was eluted with a 50–500 mM imidazole gradient, pH 7.5. The pooled NS2B–NS3 fractions, which contained a 10 mM residual imidazole concentration, were re-chromatographed on a Co2+–agarose column to generate the homogenous samples. (C) Autolytic conversion of the NS2B–NS3 precursor into NS3. The purified material (samples 1, 2 and 3) was incubated overnight (0.02–0.2 mg/ml; pH 8) at 24°C to generate the autolytically processed NS3 proteinase. (D) The representative purified sample of NS3. An antibody against the His6-tag was used in Western blotting. After the purification and autolysis steps, the yield of the purified NS3 enzyme was approx. 5 mg of protein/litre of E. coli culture. (E) Aprotinin and nona-D-Arg-NH2 inhibit autolysis of the NS2B–NS3 construct. The purified NS2B–NS3 sample (2.5 µM) was incubated alone or with aprotinin (25 µM; a 10-fold molar excess) and nona-D-Arg-NH2 (40 µM; a 16-fold molar excess) each for 16 h at 24°C. Molecular-mass sizes are given in kDa.

of NS2B-linker-NS3 were produced as a soluble protein. The soluble fraction was loaded on to a pre-equilibrated Co2+-agarose affinity column. The NS2B–NS3 protein was then eluted with a 10–500 mM gradient of imidazole concentrations (Figure 1). The pooled, partially purified, NS2B–NS3 fractions were dialysed and then re-chromatographed on a Co2+-agarose column to acquire the homogenous NS2–NS3 samples. During purification, we observed an autolytic conversion of the 36 kDa NS2B–NS3 construct into the 30 kDa NS3 proteinase. To facilitate the autolytic conversion, the purified samples (0.02–0.2 mg/ml) were incubated overnight at pH 8 and 24°C to generate the autolytically processed individual NS3 proteinase. Under these experimental conditions, complete conversion was accomplished yielding NS3 (Figure 1). The resulting NS3 samples retained the C-terminal His6-tag as the NS3 moiety was readily recognized by anti-His6-tag antibody (Figure 1). We determined that, in the course of an autolytic conversion, NS3 cleaved the EYKK↓G16DT sequence (numbering starts from the N-terminus of NS3) that generated the individual NS3 proteinase commencing from the N-terminal Gly16 (Figure 1). The catalytically inert NS2B–NS3 mutant with the H51A mutation of the active site His51 had approx. 0.1–1% of enzymatic activity when compared with that of the autolytically activated NS2B–NS3 construct, and, accordingly, this mutant was not autolytically cleaved into NS3 (results not shown). Inhibitors of NS3 completely inhibited autolysis of the NS2B–NS3 construct and its conversion into NS3 (Figure 1). The catalytically inert NS2B–NS3 mutant has already been crystallized, and the crystallographic analysis is currently in progress (A. Aleshin, S. A. Shiryaev, R. C. Liddington and A. Y. Strongin, unpublished work). According to the results of size-exclusion chromatography (results not shown), the NS2b remains non-covalently bound with the NS3 moiety after the autocatalytic cleavage of the NS2B–NS3 construct.

**Table 1**  NS3 cleaves fluorescent peptide substrates of furin

<table>
<thead>
<tr>
<th>Substrate</th>
<th>(k_{\text{cat}}/K_m) (s(^{-1}) M(^{-1}))</th>
<th>(K_m) (µM)</th>
<th>(V_{\text{max}}) (µM/s)</th>
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<td>Z-RR-AMC</td>
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<td>2</td>
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<tr>
<td>Boc-RVRR-AMC</td>
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<td>Boc-D-benzy1-EGR-pNA</td>
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**NS3 cleaves furin-like motifs in protein substrates**

In the viral polyprotein precursor, NS3 predominantly cleaves the sequence regions with positively charged amino acid residues at both the P1 and P2 positions [12]. This cleavage preference resembles that of cathepsin B and furin [5,27]. In our cleavage tests, WNV NS3 efficiently cleaved the furin fluorescent substrates Boc-RVRR-AMC and Pyr-RTKR-AMC, while the cleavage of the cathepsin B substrate Z-RR-AMC was significantly less efficient (Table 1). The cleavage of the peptide substrates followed Michaelis–Menten kinetics. The Lineweaver–Burk plot was linear over the range of the substrate concentrations.
The furin cleavage motif in the anthrax PA83 sequence is underlined. An arrow indicates an Arg167-Ser168 scissile bond in the PA83 peptide sequence (top). The NS3 protease cleaves the RKKRR↓S furin motif. This cleavage converts PA83 into the mature PA63 (bottom). Molecular-mass sizes are indicated in kDa.

To corroborate the ability of NS3 to process the substrates of furin, we analysed the cleavage of the peptide K83TRHSRRSRRSL94 that spans the furin-cleavage site in WNV prM. As anticipated, the NS3 proteinase efficiently cleaved the RKKR167↓S168TS furin cleavage motif in PA83 and generated a 941 Da K83TRHSRR digest product, thus confirming the K83TRHSRR↓SRRSRRSL94 peptide to NS3-mediated proteolysis. The mass of the digested products was identified by MS analysis of the NS3 cleavage peptides

The analysis returned a score for each identified site, based on the weighted matrix. The analysis revealed 28 top scoring hits with the score of 46–48 in the human proteome, as shown in Table 2. The identified hits were predominantly represented by the hypothetical and predicted proteins and by the proteins without any obvious physiological relevance to the NS3 function. The analysis also identified two brain proteins, which may be susceptible to NS3 proteolysis and estimated masses of the peptides. This software uses the scoring matrix rather than a position-specific scoring matrix (on a scale of −5.0 to +5.0) for the P4−P4′ cleavage positions of the known NS3 protein and peptide substrates. This software uses the scoring matrix rather than the sequence motif in searching for cleavage targets [32]. PoPS was then used to search for the presence of the NS3 cleavage profile in the human proteome (28 796 proteins). Because, in our search motif, the amino acid residues other than positively charged arginine and lysine occupied the P4−P4′ position, the substrates of furin (which contain the presence of positively charged residues at P4) would not be identified in our searches. To filter these predictions further, the programs PSIPRED and NCOILS (integrated in the PoPS system) were used to predict the secondary structure of the potential protein targets and to search for cleavage sites that are located in unstructured regions.

This analysis returned a score for each identified site, based on the weighted matrix. The analysis revealed 28 top scoring hits with the score of 46–48 in the human proteome, as shown in Table 2. The identified hits were predominantly represented by the hypothetical and predicted proteins and by the proteins without any obvious physiological relevance to the NS3 function. The analysis also identified two brain proteins, which may be relevant to the biology of WNV: MBP and myelin protein zero (PoPS score of both was 46). These proteins are required for the normal functioning of neurons and their absence or mutation is associated with neuron degeneration and neurological pathologies [33–36].
To confirm the validity of PoPS prediction, we evaluated the ability of NS3 to cleave MBP in vitro. As predicted, MBP was highly sensitive to specific cleavage by WNV NS3 (Figure 4).

**d-Arginine-based peptides and serpins are the NS3 inhibitors**

D-Arginine-based peptides and serine protease inhibitors (serpins) are potent inhibitors of furin [23,37]. Based on our data that show that NS3 has furin-like activity, we tested whether similar peptide and protein inhibitors would inhibit WNV NS3. The 6–12-mer d-arginine peptides were effective at a nanomolar range against the WNV NS3 protease in vitro (Table 3). Consistent with these findings, NS3 was also sensitive to a well-known inhibitor of furin, α1-antitrypsin variant Portland [38]. Among the protein inhibitors examined, aprotinin was most effective in inhibiting NS3 ($K_i = 26$ nM). Aprotinin and d-arginine peptides were efficient both in the inhibition of the cleavage of the fluorescence peptide model substrates by NS3 and in protecting the protein substrates from the NS3 cleavage. Thus both aprotinin and d-arginine-based peptides efficiently inhibit NS3 proteolysis of MBP in vitro (Figure 4).

Lineweaver–Burk plots of the d-arginine-based inhibitors demonstrated strictly competition-type inhibition (results not shown). A series of d-arginine inhibitors with a chain length of six to twelve residues was synthesized and tested for inhibitory potency against NS3 and furin. In agreement with the earlier data [23,37], Table 3 shows that the $K_i$ of the d-arginine peptide for furin increased from 36 to 100 nM as the chain length decreased from nine to six residues. In contrast, the $K_i$ of hexa-d-Arg-NH$_2$ for NS3 was 80–100-fold higher when compared with that of nona-d-Arg-NH$_2$ peptide. These findings suggest that there is a significant difference in the binding mode of the positively charged inhibitors with furin and with NS3. To identify this difference, we built a spatial model of WNV using a known structure of Dengue virus NS3 as a template [19,39].

**Modelling of the spatial structure of NS3**

Because the level of homology between the WNV and Dengue NS3 is high (above 50%), we could produce the model of WNV NS3 (R.M.S.D. = 0.21 Å) (Figure 5). This model was used to guide our studies until the actual crystallographic structure of NS3 becomes available. The in silico model of the NS3 protease shows a low density of negatively charged residues in the proximity of the catalytic triad and this structural relationship emphasizes the importance of the two aspartate residues (Asp$^{23}$ and Asp$^{129}$) (Figure 5). According to this model, there are no negatively charged residues, except for Asp$^{75}$ and Asp$^{129}$, in the active-site groove of WNV NS3. We suspect that these two residues of NS3 interact with the positively charged arginine-based antagonists. In contrast, the number of negatively charged residues is high in furin’s catalytic site region [27,40].

**Cell-based assays confirm the inhibitory effect of d-arginine-based peptides**

Although our in vitro data suggested that d-arginine-based peptides had the capacity to inhibit NS3 protease activity, it was important to confirm the relevance of this finding. To assess this, we treated primary neurons with increasing concentrations of nona-d-Arg-NH$_2$ and then infected them with the pathogenic New York strain of WNV. At a 10–50 μM range of peptide, a several log reduction of virus production was observed (Figure 6). There was no significant non-specific toxicity in the assays. The relatively high concentrations of d-arginine peptides that were required (compared with the $K_i$) may be explained by the poor
and by aprotinin (K_i of 100–1000 nM; Table 3). The data show that NS3 (2 µM) is fully inhibited at a 1–2 nM concentration. However, the K_i value of the inhibitors was determined using Boc-RVRR-AMC as a substrate. Note the dramatic difference in the efficiency of inhibition of furin and NS3 by nona-0-Ang-NH2 and hexa-0-Ang-NH2.

As with bacterial proteinases (subtilisin and thermolysin) and mammalian furin [27,41–44], the viral NS2B, most probably stabilizes the folding intermediates [45–48] on the path to the fully folded/active catalytic domain of NS3. Because the individual NS3 was catalytically active in the peptide and protein cleavage assays, we believe that the presence of the covalently bound NS2B sequence is not required for the enzymatic activity of NS3.

Our studies determined that NS3 exhibited furin-like, albeit less restricted, cleavage preferences. These distant relationships between furin and NS3 led us to hypothesize that there are common inhibitors of these proteinases. This hypothesis facilitated the identification of the inhibitors of NS3. Thus the D-arginine-based peptides are nanomolar range inhibitors of NS3 in vitro.

Based on the modeling of the three-dimensional structure of WNV NS3, we suggest that the negatively charged Asp75 and Asp129 residues, which are 20 Å apart, appear to be important for the interactions with the D-arginine-based inhibitors, which should span approx. 20 Å to reach both Asp75 and Asp129. It is obvious that D-arginine-based inhibitors will interact most efficiently with the aspartate residues and especially with the partially protonated Asp75 of the catalytic triad only at high pH. These considerations were taken into account in our inhibitory studies, which were performed at pH 8.

We hypothesized that the presence of Asp75 and Asp129 could explain why short arginine peptides are less efficient inhibitors of NS3 as compared with furin. The synthesis and the testing of these structure-based inhibitors are currently in progress. Because we have already prepared the crystals of the catalytically inert H51A NS2B–NS3, we will soon know the exact role of and the precise distance between Asp75 and Asp129. We believe that these studies at atomic resolution level will shed light on the mechanism of NS3 inhibition by the positively charged arginine peptides as well as guiding the design of the synthetic, non-peptidic, inhibitors.

Knowledge of the substrate preference of NS3 led us to search for possible host targets of NS3 proteolysis. Using a prediction program, we identified MBP as an NS3 cleavage target. Experimental studies confirmed that MBP was specifically

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Figure 5 Model of the WNV NS3 proteinase

The numbering corresponds to the WNV NS3 sequence. The residues of the catalytic triad are purple. The surface residues conserved in all of mosquito-borne flaviviral NS3 proteinases are light blue. The residues conserved in the WNV, Usutu virus, Japanese encephalitis virus, Murray Valley encephalitis virus and Kunjin virus NS3 are red. These residues are not conserved in Dengue virus and yellow fever virus NS3. The corresponding residues of the Dengue virus NS3 are shown in parentheses. Modelling was performed using the Dengue NS3 protease (PDB code 1DF9) as a template. The model total energy score is $-0.0379$ (local $= -0.0524$, burrial $= -0.0013$, contact $= 0.0159$), which is consistent with the feasible molecular models. The R.M.S.D. of the model is 0.21 Å.

Figure 6 Nona-$\epsilon$-Arg-$\text{NH}_2$ reduces WNV infection in primary neurons

SCG neurons from wild-type mice infected with WNV at a multiplicity of infection of 1. Neurons were treated with increasing concentrations of nona-$\epsilon$-Arg-$\text{NH}_2$ 2 h before infection. At 24 h after infection, supernatants were harvested, and the amount of infectious WNV was titrated by viral plaque assay on BHK21 cells. The assay was performed in triplicate. Results are means ± S.E.M.

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